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# Specific Gut Commensal Flora Locally Alters T Cell Tuning to Endogenous Ligands

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## SUMMARY

**Differences in gut commensal flora can dramatically influence autoimmune responses, but the mechanisms behind this are still unclear. We report, in a Th1-cell-driven murine model of autoimmune arthritis, that specific gut commensals, such as segmented filamentous bacteria, have the ability to modulate the activation threshold of self-reactive T cells. In the local microenvironment of gut-associated lymphoid tissues, inflammatory cytokines elicited by the commensal flora dynamically enhanced the antigen responsiveness of T cells that were otherwise tuned down to a systemic self-antigen. Together with subtle differences in early lineage differentiation, this ultimately led to an enhanced recruitment of pathogenic Th1 cells and the development of a more severe form of autoimmune arthritis. These findings define a key role for the gut commensal flora in sustaining ongoing autoimmune responses through the local fine tuning of T-cell-receptor-proximal activation events in autoreactive T cells.**

## INTRODUCTION

Commensal flora can have profound effects on the adaptive immune response and, in particular, on the development and plasticity of gut-associated T helper (Th) and T regulatory (Treg) cell subsets (Mazmanian et al., 2005; Gaboriau-Routhiau et al., 2009; Cerf-Bensussan and Gaboriau-Routhiau, 2010; Round and Mazmanian, 2010; Atarashi et al., 2011; Chung et al., 2012). In mice, a single species of the gut microflora, segmented filamentous bacteria (SFB), promotes the generation of an intestinal subset of Th17 cells and enhances the expression of interferon-γ (IFN-γ) or Foxp3 in gut T cell subsets (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009). When properly controlled, such dialog participates in enhancing host protection against external pathogens, such as *Citrobacter rodentium* (Ivanov et al., 2009). In the context of a susceptible genetic background, however, SFB can influence the development of autoimmune pathologies, as demonstrated in murine models of rheumatoid arthritis (Wu et al., 2010) and multiple sclerosis (Lee et al., 2011). In both cases, the exact mechanism(s) involved in the coordination of T cell responses by proinflammatory members of the gut microflora remain poorly understood. Furthermore, analyzing the effect of the gut commensal flora solely on the basis of T cell differentiation can't explain the broad effect that SFB can have on gut-associated immune responses (Talham et al., 1999; Gaboriau-Routhiau et al., 2009; Chung et al., 2012).

The case of T cell responses directed against self-antigen expressed in the gut and gut-associated lymphoid organs adds an additional layer of complexity. In various settings of chronic antigenic stimulation, T cells have been shown to rapidly lose their proliferative and effector potential (Carmichael et al., 1993; Rehermann et al., 1996; Oxenius et al., 1998; Zajac et al., 1998; Brooks et al., 2005). Detailed studies in double transgenic models have demonstrated the intrinsic property of mature $CD4^+$ T cells to tune their functional avidity to the level of ambient antigenic stimulation (Tanchot et al., 2001; Singh and Schwartz, 2003), as predicted by the tunable-activation-threshold model (Grossman and Paul, 1992, 2001). In contrast to in vitro clonal anergy, this tuning of T cell sensitivity, also known as adaptive tolerance, requires persistent antigenic expression and is partially reversible upon removal of the antigen (Tanchot et al., 2001; Han et al., 2010). From a molecular point of view, this unresponsive state reflects an impairment of T cell receptor (TCR)-proximal signaling (Schwartz, 2003; Choi and Schwartz, 2007, 2011), a block that can be bypassed by direct stimulation of the T cells with phorbol myristate acetate (PMA) and ionomycin along with fresh antigen-presenting cells (APCs) (Chiodetti et al., 2006). Given that this T-cell-intrinsic tolerance mechanism represents one of the first checkpoints in the establishment of a chronic self-specific T cell response, an impact of proinflammatory members of the gut microbiota at the level of T cell tuning could explain their potential in driving such a broad spectrum of autoimmune diseases.

We report here an example of Th1-cell-driven autoimmune arthritis for which disease incidence and severity are crucially linked to a specific flora, and this correlates with the colonization of hosts by SFB. By carefully analyzing the impact of the gut microbiota on the highly coordinated, chronic, self-specific T cell response observed in this in vivo model, we show that proinflammatory signals elicited by specific commensals have the ability to lower the activation threshold of T cells facing chronic antigenic stimulation in the local microenvironment

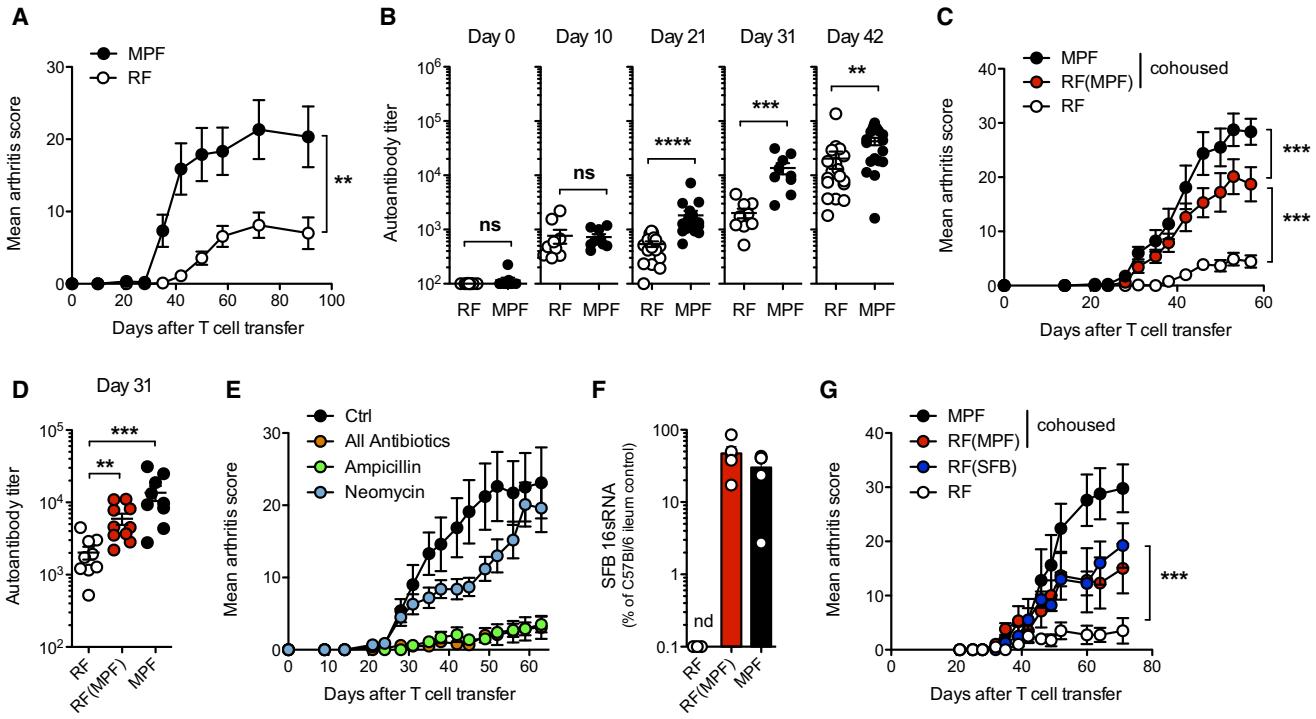


**Figure 1. SFB Colonization of Host Gut Commensal Flora Enhances the Severity of Autoimmune Disease in a T-Cell-Transfer Model of Arthritis**

(A and B) Weekly arthritis score (A) and serum autoantibody titers (B) in RF- or MPF-housed mPCC,*Cd3e*$^{-/-}$ hosts at indicated time points after naive 5C.C7 T cell transfer. Data (mean ± SEM) were pooled from two (A) and four (B) independent experiments (n = 9 and 18 mice per group, respectively).

(C and D) One group of RF-housed mPCC,*Cd3e*$^{-/-}$ mice was cohoused with MPF-housed hosts for 3 weeks prior to T cell transfer (RF(MPF)). Biweekly arthritis scores (C) and autoantibody titers at day 31 (D) are shown. Data (mean ± SEM) were pooled from two independent experiments (n = 9 for RF, n = 10 for RF(MPF), and n = 8 for MPF).

(E) Various antibiotic formulations were given in the drinking water of MPF-housed mPCC,*Cd3e*$^{-/-}$ mice starting 3 weeks prior to T cell transfer and were maintained for the length of the experiment. The "all antibiotics" formulation contained ampicillin, neomycin, vancomycin, and metronidazole. Data (mean ± SEM) were pooled from two independent experiments (n = 10 mice per group).

(F) The amount of SFB 16sRNA in cecal contents of indicated mPCC,*Cd3e*$^{-/-}$ hosts 8 weeks after T cell transfer (n = 5 mice per group; mean ± SEM).

(G) Same as (C) with an additional group of RF-housed mPCC,*Cd3e*$^{-/-}$ mice cohoused with SFB-monocolonized germ-free mice 3 weeks prior to T cell transfer (RF(SFB)) (n = 4 for RF and RF(SFB), n = 6 for RF(MPF), and n = 5 for MPF; mean ± SEM).

The following abbreviations are used: ns, not significant; and nd, not detected. See also Figure S1.

of the gut-draining lymphoid tissues. By bypassing and/or reversing T-cell-intrinsic tuning mechanisms, microbiota-induced signals favor chronic activation of self-reactive T cells and magnify early effects of the gut flora on distal parameters, such as lineage differentiation. This work provides new insights into the sequence of events locally triggered by proinflammatory members of the gut microbiota to promote systemic autoimmune diseases.

## RESULTS

### SFB Regulate the Severity of Autoimmune Disease in a T Cell Transfer Model of Arthritis

We previously reported that transfer of 5C.C7 TCR-transgenic *Rag2*$^{-/-}$ CD4$^{+}$ T cells (expressing a $V_{\alpha}11V_{\beta}3$ TCR specific to pigeon cytochrome *c* [PCC]) into a T-cell-deficient mPCC, *Cd3e*$^{-/-}$ host leads to polyclonal hypergammaglobulinemia associated with mild arthritis (Singh et al., 2006). We noticed a delayed incidence and a decreased severity of the arthritis when our colony of mPCC,*Cd3e*$^{-/-}$ hosts, originally housed in a Murine Pathogen Free (MPF) barrier in Taconic Farms, was rederived, colonized with the sole Altered Schaedler Flora, and transferred to a separate Restricted Flora (RF) barrier.

Side-by-side experiments carefully monitoring the appearance of arthritis in RF- or MPF-housed hosts substantiated our initial observations. RF-housed hosts developed less-severe arthritis (Figure 1A), and the first symptoms appeared on average 2 weeks later than in their MPF-housed counterparts. This was also reflected in the emergence of anti-cellular antibodies in the sera, where the late qualitative change in the autoantibody response, detectable by day 21 in MPF-housed hosts, was delayed by about 10 days in RF-housed hosts (Figure 1B). Cohousing mPCC,*Cd3e*$^{-/-}$ hosts of RF and MPF origin for 3 weeks was sufficient to transfer much of the enhanced susceptibility to arthritis to mice originally raised in an RF microenvironment (cohoused-RF(MPF)) (Figures 1C and 1D). This confirmed that a transmissible factor from the MPF microenvironment, and not a genetic drift and selection linked to our recent rederivation,

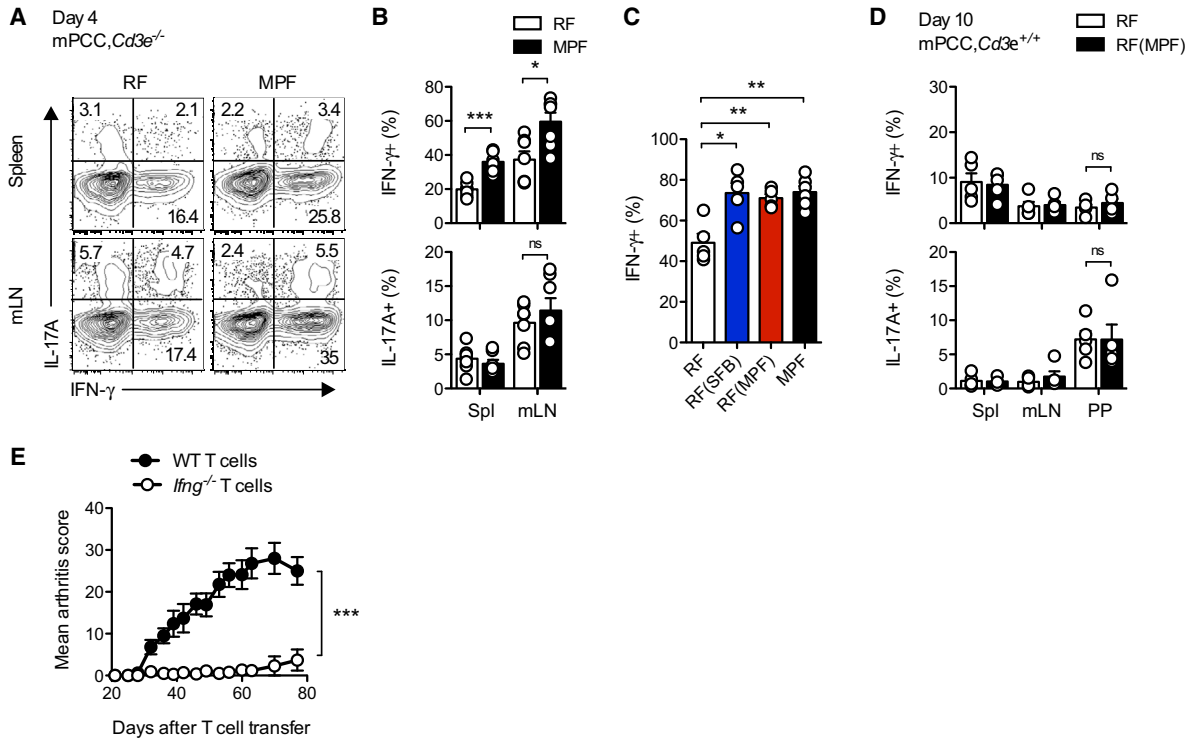


**Figure 2. SFB-Containing Flora Promotes the Differentiation of Arthritis-Inducing Th1 Cells**

(A–C) Four days after transfer of naive 5C.C7 T cells into mPCC,*Cd3e*$^{-/-}$ hosts, the cells were stained for intracellular IL-17A and IFN-γ after a 3 hr stimulation with PMA and ionomycin. Representative IFN-γ and IL-17A expression profiles, gated on live CD4$^+$Vβ3$^+$ T cells, are shown in (A), and the frequencies of IFN-γ$^+$ or IL17A$^+$ in live CD4$^+$Vβ3$^+$ T cells in the indicated organs are shown in (B). The frequencies of IFN-γ$^+$ in live CD4$^+$Vβ3$^+$ T cells in mLNs of the indicated host are shown in (C). Data (mean ± SEM) were pooled from three independent experiments each (n = 7 mice per group in A and B, and n = 5–6 mice per group in C).

(D) Ten days after transfer of naive CD45.1$^+$ 5C.C7 T cells into 8- to 12-week-old mPCC,*Cd3e*$^{+/+}$ hosts, the cells were stained for intracellular IL-17A and IFN-γ after a 5.5 hr stimulation with PMA and ionomycin. The frequencies of IFN-γ$^+$ or IL17A$^+$ in live CD45.1$^+$ CD4$^+$Vβ3$^+$ T cells in indicated organs are shown. Data (mean ± SEM) were pooled from five independent experiments, each representing one pool of six RF-housed or two cohoused-RF(MPF) mice. RF(MPF) hosts were cohoused for 2 weeks with MPF-housed mPCC,*Cd3e*$^{-/-}$ mice prior to T cell transfer.

(E) Biweekly arthritis scores of MPF-housed mPCC,*Cd3e*$^{-/-}$ mice after transfer of naive 5C.C7 wild-type or *Ifng*$^{-/-}$ 5C.C7,*Rag2*$^{-/-}$ T cells. Data (mean ± SEM) were pooled from two independent experiments (n = 10 mice per group).

The following abbreviation is used: ns, not significant. See also Figure S2.

was having a dominant impact on the outcome of the autoimmune response.

To better characterize the nature of such a factor, we next treated MPF-housed hosts with a cocktail of four antibiotics (ampicillin, neomycin, vancomycin, and metronidazole), previously described to ablate most of the gut commensal bacterial species (Ivanov et al., 2008). This appeared sufficient to almost fully prevent the appearance of any sign of autoimmune arthritis (Figure 1E). Ampicillin alone, but not neomycin sulfate, led to a similar outcome. This suggested a key role for specific, ampicillin-sensitive members of the MPF gut commensal flora.

A similar impact of an ampicillin-sensitive member of the intestinal microbiota, SFB, has recently been reported in the K/BxN spontaneous model of rheumatoid arthritis (Wu et al., 2010). In light of this observation, we tested for and found SFB 16sRNA by quantitative PCR in both ileum and cecum of 6-week-old MPF-housed mPCC,*Cd3e*$^{-/-}$ hosts, but not RF-housed hosts, at a level similar to that of control MPF-housed C57BL/6NTac mice from Taconic Farms (Ivanov et al., 2009) (Figure S1A, available online). This commensal organism was easily transferred to cohoused-RF(MPF) hosts (Figure 1F) and was absent from mice undergoing ampicillin, but not neomycin sulfate, treatment (Figure S1B). Finally, cohousing of RF-housed hosts with SFB-monocolonized germ-free mice (cohoused-RF(SFB)) demonstrated the critical impact that the addition of only SFB to the host microbiota can have on the outcome of the autoimmune arthritis in this model (Figure 1G).

## SFB Promote the Differentiation of Arthritis-Inducing Th1 Cells

The impact of SFB on autoimmune responses has been previously linked to its ability to promote interleukin-17A (IL-17A)-producing CD4$^+$ T helper 17 (Th17) cells, as well as T follicular helper (Tfh) cells, in gut-associated lymphoid tissues and spleen (Wu et al., 2010; Lee et al., 2011). In our model, however, no significant difference could be detected for the early differentiation of autoreactive IL-17A$^+$ 5C.C7 T cells (Figures 2A and 2B) or PD-1$^+$Bcl-6$^+$ 5C.C7 Tfh cells (Figure S2A) and germinal center B cells (Figures S2B and S2C) in either mesenteric lymph nodes (mLNs) or the spleen of mPCC,*Cd3e*$^{-/-}$ hosts housed under

RF or MPF conditions. There were also no notable changes in Foxp3$^+$ T cells, which were close to undetectable in both hosts by day 4 in any organ we looked at (Figure S2D and data not shown), as previously reported (Singh et al., 2006). IL-10 production by CD4$^+$ T cells also appeared similar in mLNs of RF- and MPF-housed hosts at day 4 and actually increased in the spleen of MPF-housed hosts (Figures S2E and S2F), supporting the idea that the RF commensal flora did not simply regulate the balance between proinflammatory and anti-inflammatory T cell responses.

Instead, a reproducible increase in the frequency of IFN-$\gamma^+$ 5C.C7 T cells (Th1 cells) was detectable in both the spleen and mLNs of MPF-housed, as well as cohoused-RF(SFB) and -RF(MPF), hosts (Figures 2A–2C). Interestingly, a large majority of the IL-10-producing T cells coexpressed IFN-$\gamma$ (Figure S2E), reminiscent of the IL-10-producing Th1 cells described in other models of Th1-driven chronic infections and autoimmunity (Anderson et al., 2007; Jankovic et al., 2007; Gabrysová et al., 2009). Confirming our observations in T-cell-deficient hosts, SFB-independent differentiation of IL-17A$^+$ 5C.C7 T cells was also observed after transfer into RF-housed T-cell-replete mPCC,*Cd3e*$^{+/+}$ hosts, although it was slightly delayed and limited to Peyer's patches (PPs) (Figure 2D and data not shown). The differentiation of IFN-$\gamma^+$ 5C.C7 T cells appeared, however, fully blunted, as previously described and in line with the absence of autoimmune pathologies (Singh et al., 2006).

The importance of the Th1 cell component of the T cell response in driving the appearance of autoimmune pathologies was further investigated with the use of *Ifng*$^{-/-}$ 5C.C7 T cells. Transfer of these cells into MPF-housed mPCC,*Cd3e*$^{-/-}$ hosts resulted in greatly reduced arthritis compared to that observed with wild-type 5C.C7 T cells (Figure 2E). This result confirms an essential role for this proinflammatory cytokine in our T-cell-transfer model of autoimmune arthritis. More importantly, by demonstrating a key role for SFB in enhancing a Th1-cell-driven autoimmune disease, these results suggest that SFB have a much broader impact on autoimmune responses than the sole control of Th17 T cell differentiation.

## SFB Sustain Chronic Proliferation of Self-reactive CD4$^+$ T Cells in Gut-Associated Lymphoid Tissues

SFB induce the full maturation of gut-associated Th cell responses (Gaboriau-Routhiau et al., 2009; Chung et al., 2012). Analyzing polyclonal CD4$^+$ T cell populations in RF- and MPF-housed mPCC,*Cd3e*$^{+/+}$ hosts, we also found a clear accumulation of polyclonal Th17 and Th1 cells in lamina propria (LP) and PPs of the small intestine in MPF-housed hosts (Figure S2G), in line with the colonization of the MPF gut commensal flora by SFB. This was further associated with a marked increase in TCR$\beta^+$CD4$^+$ T cell number (Figure S2H), correlating with an enhanced proliferation of such cells, as revealed by Ki-67 expression and 5-ethynyl-2'-deoxyuridine (EdU) incorporation (Figures S2I and S2J). These results thus prompted us to investigate a potential impact of SFB-derived signals on autoreactive T-cell-proliferative responses in gut-associated lymphoid tissues.

Echoing observations made under steady-state homeostasis, we observed a sustained lymphadenopathy of MPF-housed mPCC,*Cd3e*$^{-/-}$ host mLNs, as well as a slight splenomegaly following 5C.C7 T cell transfer (data not shown). In line with the overall size of the mLNs, a 12- to 50-fold increase in accumulation of 5C.C7 T cells was detected as early as day 14 in mLNs and the LP of the small intestine in MPF-housed hosts compared to RF-housed hosts (Figure 3A). Detailed kinetic analysis did not reveal any difference in the primary proliferation phase occurring in either host up to day 4 (Figure S3A). However, the secondary, chronic, expansion phase was dramatically curtailed in RF-housed mPCC,*Cd3e*$^{-/-}$ hosts in both the spleen and mLNs. No significant differences in absolute T cell numbers were observed between the two hosts in peripheral lymph nodes (pLNs) up to day 57 (data not shown).

In favor of a second wave of active in vivo proliferation of chronically stimulated 5C.C7 T cells, a secondary peak of Ki-67 expression, correlating with strong EdU incorporation, was detected around day 14 in all gut-associated lymphoid tissues (Figures 3B and 3C and S3B and S3C). In line with the incidence of arthritis in these hosts, the enhanced proliferation and accumulation of 5C.C7 T cells was recapitulated by the sole addition of SFB to the RF flora (Figure 3D and data not shown). This secondary phase of T cell proliferation was additionally associated with signs of sustained T cell activation, as evidenced by the late re-expression of the high-affinity IL-2R (CD25) by a subpopulation of mLN T cells recovered from MPF-housed mPCC,*Cd3e*$^{-/-}$ hosts (Figures S3B–S3D). Expression of CD25 correlated with high expression of Ki-67 (Figure 3E) and an increased level of endogenous mTOR activation, as revealed by ex vivo analysis of S6 ribosomal protein phosphorylation (Figure 3F).

Of interest, the impact of the gut commensal flora on autoreactive T cell proliferative responses appeared to extend beyond the sole context of the lymphopenic host. Although cell numbers recovered from mPCC,*Cd3e*$^{+/+}$ hosts were dramatically lower than those recovered from mPCC,*Cd3e*$^{-/-}$ hosts (as previously described by Singh et al., 2006) and close to the limit of detection in the LP of both hosts, a clear accumulation of transferred CD45.1$^+$ 5C.C7 T cells was observed in the PPs of MPF-housed mPCC,*Cd3e*$^{+/+}$ hosts (Figures 3G and S3E). This also correlated with enhanced proliferation (Figures 3H and 3I). Under these additional T-cell-extrinsic constraints, however, no difference in cell numbers recovered from either the spleen or mLNs was observed up to day 10. Similar results were obtained in adult RF-housed hosts cohoused for 2 weeks with MPF-housed mPCC,*Cd3e*$^{-/-}$ hosts prior to T cell transfer (Figure 3J). All together, our results demonstrate a nonredundant role for specific members of the MPF commensal flora, such as SFB, in promoting chronic activation and proliferation of autoreactive T cells in gut-associated lymphoid tissues.

## Gut Microbiota Modulates T Cell Tuning to Self-antigen in Gut-Associated Lymphoid Tissues

Previous data from this experimental model indicate that the main constraint on chronic T cell activation is the result of a T-cell-intrinsic adaptation of its activation threshold to the ambient level of antigen presentation in the host (Singh and Schwartz, 2003; Singh et al., 2006). The uncontrolled chronic T cell activation observed here could therefore reflect an inadequate and/or impaired tuning of 5C.C7 T cell sensitivity

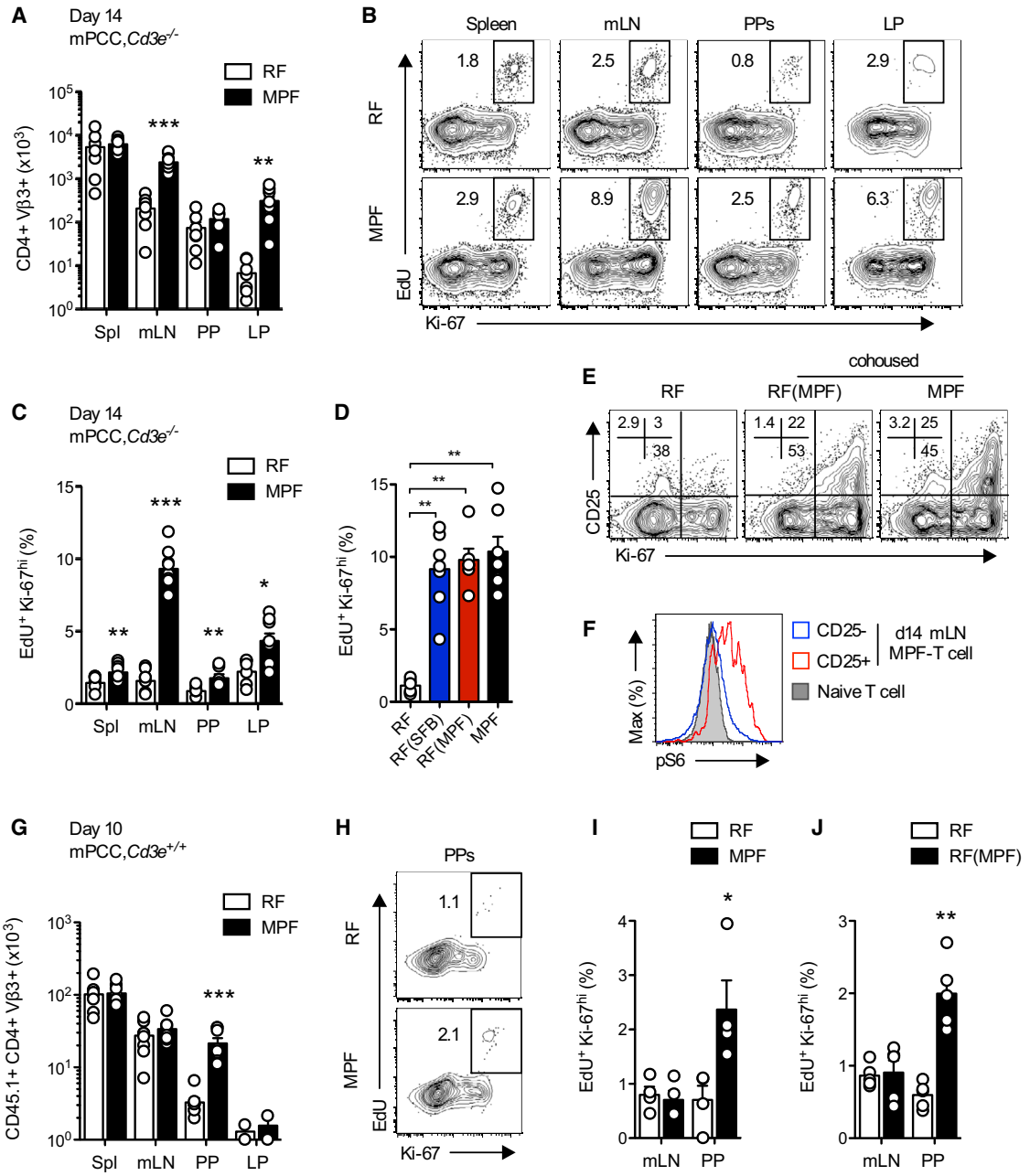


**Figure 3. SFB-Containing Flora Sustains Chronic Proliferation of Self-reactive CD4$^+$ T Cells**

(A–D) Fourteen days after naive 5C.C7 T cell transfer, mPCC,*Cd3e*$^{-/-}$ hosts were injected with EdU and sacrificed 1 hr later. The absolute number of CD4$^+$Vβ3$^+$ T cells in the spleen, mLNs, PPs, and LP of the small intestine is shown in (A). Representative EdU and Ki-67 expression profiles are shown in (B), and the frequency of EdU$^+$Ki-67$^{hi}$ cells in live CD4$^+$Vβ3$^+$ T cells isolated from the indicated organs of RF- or MPF-housed hosts (B and C) or mLNs of indicated hosts (D) is shown in (C) and (D). Data (mean ± SEM) were pooled from three independent experiments each (n = 8 mice per group in A–C, and n = 6–7 mice per group in D).

(E and F) Representative CD25 and Ki-67 expression profiles, gated on CD4$^+$Vβ3$^+$ T cells, are shown in (E), and a representative expression profile for phosphorylated S6 ribosomal protein (pS6) in CD25$^-$ (open, blue line) and CD25$^+$ (open, red line) CD4$^+$Vβ3$^+$ T cells isolated from mLNs of the indicated hosts at day 14 and compared to naive 5C.C7 T cells (closed, gray) is shown in (F).

(G–I) Ten days after transfer of naive CD45.1$^+$ 5C.C7 T cells into 4- to 5-week-old mPCC,*Cd3e*$^{+/+}$ hosts, the recipient mice were injected with EdU and sacrificed 1 hr later. The absolute number of CD45.1$^+$CD4$^+$Vβ3$^+$ T cells recovered at day 10 from indicated organs is shown in (G). Representative EdU and Ki-67 expression profiles are shown in (H), and the frequency of EdU$^+$Ki-67$^{hi}$ cells in live CD45.1$^+$CD4$^+$Vβ3$^+$ T cells isolated from mLNs and PPs is shown in (I). Data (mean ± SEM) were pooled from six (G) and four (H and I) independent experiments (n = 6 pools of 1–3 mice per group and 4 pools of 3 mice per group, respectively). All EdU and Ki-67 stainings were performed on purified CD4$^+$ T cells.

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to the ambient level of PCC presentation in the mLNs of MPF-housed hosts.

An inadequate tuning could result from a local, commensal-flora-mediated increase in TCR stimulation, e.g., through cross-reactivity to bacterial antigens or increased self-antigen presentation. However, transfer of naive 5C.C7 T cells labeled with carboxyfluorescein succinimidyl ester into MPF- or RF-housed *Cd3e*$^{-/-}$ hosts did not reveal any differences in homeostatic expansion in the absence of PCC antigen, making it unlikely that 5C.C7 TCRs cross-react to bacterial antigens specifically present in the MPF microflora (Figure S4A). Similarly, ex vivo analysis of the antigen-presenting capacity of various APCs isolated from RF- or MPF-housed mPCC,*Cd3e*$^{-/-}$ hosts did not indicate any significant changes in endogenous PCC presentation levels. Seven days after the initial T cell transfer, the ability to efficiently present endogenous PCC peptide to CD4$^+$ T cells appeared mostly restricted to CD11c$^+$ dendritic cells (DCs) in both hosts, and all DC populations tested demonstrated an equal potential to stimulate the in vitro proliferation of a population of preactivated 5C.C7 T cells (Figure S4B) or the overnight activation of chronically stimulated 5C.C7 T cells purified from the mLNs (mLN T cells) of RF- or MPF-housed hosts at day 7 (Figures S4C and S4D).

In contrast, the response of MPF mLN T cells to both sources of DCs appeared significantly enhanced as compared to the response of RF mLN T cells (Figures S4C and S4D). Consistent with an impaired tuning of T cells residing in the mLNs of MPF-housed hosts, in vitro activation of MPF mLN T cells with moth cytochrome *c* (MCC)-peptide-pulsed naive *Cd3e*$^{-/-}$ splenocytes revealed a selectively increased sensitivity of these cells in both their ability to upregulate CD69 (Figures 4A and 4B) and to proliferate (Figures 4C and 4D). Similar results were obtained with plate-bound anti-CD3 stimulation in the absence of any added APCs (Figure 4E). Sensitivity of MPF mLN T cells was never fully restored to the level of naive T cells, yet these cells displayed a distinctively lower activation threshold to MCC peptide than did RF mLN, RF spleen, or MPF spleen T cells.

By primarily affecting the TCR proximal signaling machinery, particularly Zap70-mediated phosphorylation of LAT (Choi and Schwartz, 2007, 2011), T cell tuning results in the downregulation of all functions downstream of TCR signaling, particularly IL-2 production (Tanchot et al., 2001). Accordingly, and as early as day 7 after initial T cell transfer, less than 3% of 5C.C7 T cells recovered from either the spleen or mLNs of RF-housed mPCC,*Cd3e*$^{-/-}$ hosts displayed IL-2 production capacity after 3 hr of in vitro activation with a high dose of MCC peptide (3 μM) (Figure 4F). In comparison, such potential appeared clearly greater in both spleen and mLN T cells isolated from MPF-housed hosts. This differential IL-2 production appeared maximal around day 14 (Figures 4F and 4G), thus mimicking the kinetics observed for CD25 and Ki-67 expression. More importantly, it persisted up to day 28, arguing for a stable state of impaired tuning of such cells rather than a delayed induction of T-cell-intrinsic tolerance. As a control, stimulation of T cells with PMA and ionomycin, which can bypass the block in proximal TCR signaling normally associated with T cell tuning (Chiodetti et al., 2006), gave comparable IL-2 responses from all the populations examined (Figures 4F and 4G). In this type of assay, the potential of tuned T cells to produce IL-2 is thus best represented as a ratio between the frequency of IL-2 producers obtained under MCC-peptide-mediated stimulation and the frequency of IL-2 producers obtained under PMA- and ionomycin-mediated stimulation; this ratio is subsequently referred to as the tuning ratio. Such analysis revealed that, at any time point studied after day 7, less than 5% of the IL-2-competent RF mLN T cells appeared to still be capable of making IL-2 after TCR-mediated activation (Figure 4H and data not shown). In contrast, up to 20%–30% of the MPF, cohoused-RF(SFB), or cohoused-RF(MPF) mLN T cells demonstrated such potential by day 14. Overall, these data demonstrate that specific members of the gut commensal flora can oppose the ability of self-reactive T cells to fully tune their activation threshold to the level of self-antigen presented in gut-associated lymphoid tissues.

## Modulation of T Cell Tuning by SFB Dynamically Affects All Self-reactive CD4$^+$ T Helper Subsets

Similar to IL-2 production, both IL-17A and IFN-γ production upon MCC peptide restimulation were higher in MPF mLN T cells, as early as day 7 and up to day 28, than in RF mLN T cells (Figures 5A and 5B). This can be partially explained by differences in Th1 cell differentiation and Th17 cell maintenance, as measured upon PMA- and ionomycin-mediated stimulation (Figures 5A and 5B). In a comparison of the tuning ratio for each individual cytokine, however, the frequency of differentiated IFN-γ$^+$ Th1 and IL-17A$^+$ Th17 cells capable of producing their respective cytokines after TCR-mediated activation increased 2.8-fold and 2.4-fold, respectively, in MPF mLNs (Figure 5C). These results confirm that regulation of T cell tuning acts as a second layer of regulation on self-reactive T cell effector functions to complement lineage differentiation, a layer partially removed in mLN T cells of SFB-harboring hosts. Of interest, T cell tuning appeared further reduced in T cells undergoing active in vivo proliferation in these organs (Figures 5D and 5E), suggesting heterogeneity of T cell tuning at the single-cell level.

On a molecular basis, the phenomena we observed could be the consequence of active T cell signaling occurring either at the early stages of T cell priming (and preventing later tuning to chronic self-antigen) or late during the chronic phase of the autoimmune response (and bypassing and/or negatively regulating already established tuning in chronically stimulated T cells). To assay the full extent of the plasticity of T cell tuning in vivo, we purified T cells from mLNs of RF-housed mPCC, *Cd3e*$^{-/-}$ hosts 7 days after the initial transfer, retransferred them into an MPF-housed host (itself pretransferred with congenic CD45.1$^+$ 5C.C7 T cells), and then analyzed their "new" responsiveness 7 days later (Figure 6A). Ex vivo restimulation revealed similarly high responsiveness of both T cell populations

(J) The same as in (I) in 8- to 12-week-old RF-housed mPCC,*Cd3e*$^{+/+}$ mice cohoused for 2 weeks with MPF-housed mPCC,*Cd3e*$^{-/-}$ mice prior to T cell transfer. Data (mean ± SEM) were pooled from five independent experiments, each representing one pool of six RF-housed or two cohoused-RF(MPF) mice. All EdU and Ki-67 stainings were performed on purified CD4$^+$ T cells.
See also Figure S3.

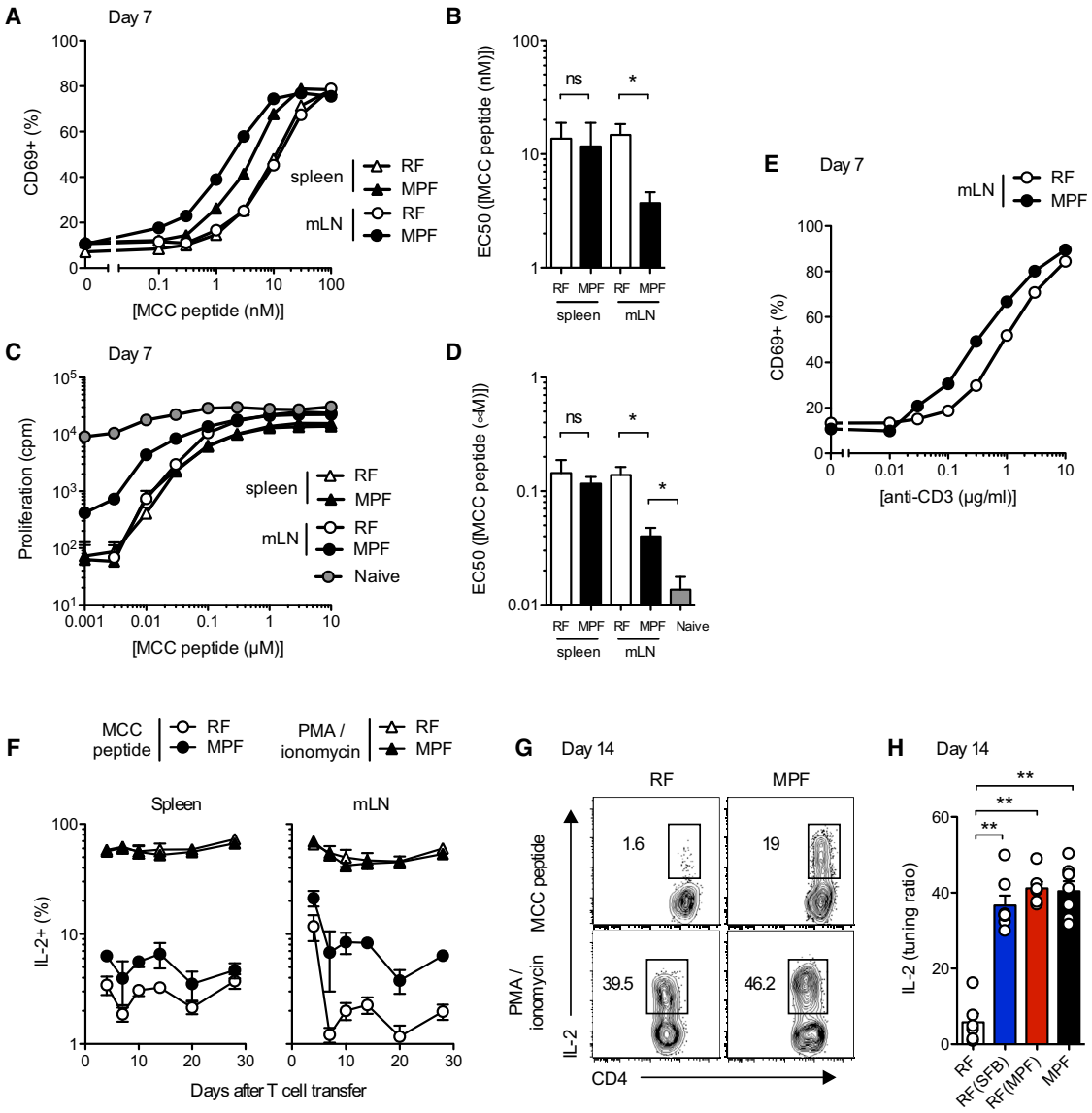


**Figure 4. SFB-Containing Flora Locally Modulates T-Cell-Intrinsic Tuning of TCR Activation Threshold to Endogenous Antigen**

(A–E) CD69 expression (A, B, and E) and 3H-thymidine incorporation (C and D) in CD4$^+$Vβ3$^+$ T cells purified from the spleen or mLNs of mPCC,*Cd3e*$^{-/-}$ hosts 7 days after naive 5C.C7 T cell transfer and cultured for 16 hr (A, B, and E) or 84 hr (C and D) with fresh *Cd3e*$^{-/-}$ splenocytes and various concentrations of MCC peptide (A–D) or plate-bound anti-CD3 (2C11) and soluble anti-CD28 in the absence of added APC (E). 3H-thymidine was added to the culture for the last 24 hr (C and D). One representative experiment (A and C) and the summary of EC50 for each independent T cell population tested (B and D) are displayed. Data (mean ± SEM) were pooled from five (A and B) and three (C and D) independent experiments; *p < 0.05, Student t test, in (D).

(F) Frequencies of IL-2$^+$ cells in live CD4$^+$Vβ3$^+$ T cells isolated from the spleen or mLNs of mPCC,*Cd3e*$^{-/-}$ hosts at the indicated time points after T cell transfer and restimulated for 3 hr with PMA and ionomycin or 3 μM MCC peptide.

(G and H) Representative expression profiles (G) and tuning ratios, as described in the Experimental Procedures, for IL-2 in live CD4$^+$Vβ3$^+$ T cells isolated at day 14 in mLNs of the indicated host (H). Data (mean ± SEM) were pooled from five independent experiments (n = 2–7 mice per time point) (F) and three independent experiments (n = 6–7 mice per group) (H).

The following abbreviation is used: ns, not significant. See also Figure S4.

as measured by IL-2 (Figure 6B). This was further associated with restored proliferation and effector functions as measured by Ki-67 expression and IFN-γ production. A more complete panel of T-cell-transfer experiments confirmed this to be true regardless of the T cell's organ of origin (Figure 6B). Furthermore, the opposite experiment, with MPF-originated T cells transferred into RF-housed hosts, gave all low responsiveness, i.e., transferred MPF T cells showed restored tuning 7 days later to the same extent as endogenous RF-T cells (Figure 6B). Overall, these results demonstrate that tuned T cells can dynamically adapt to a change in the local microenvironment by either downregulating or upregulating their TCR activation threshold.

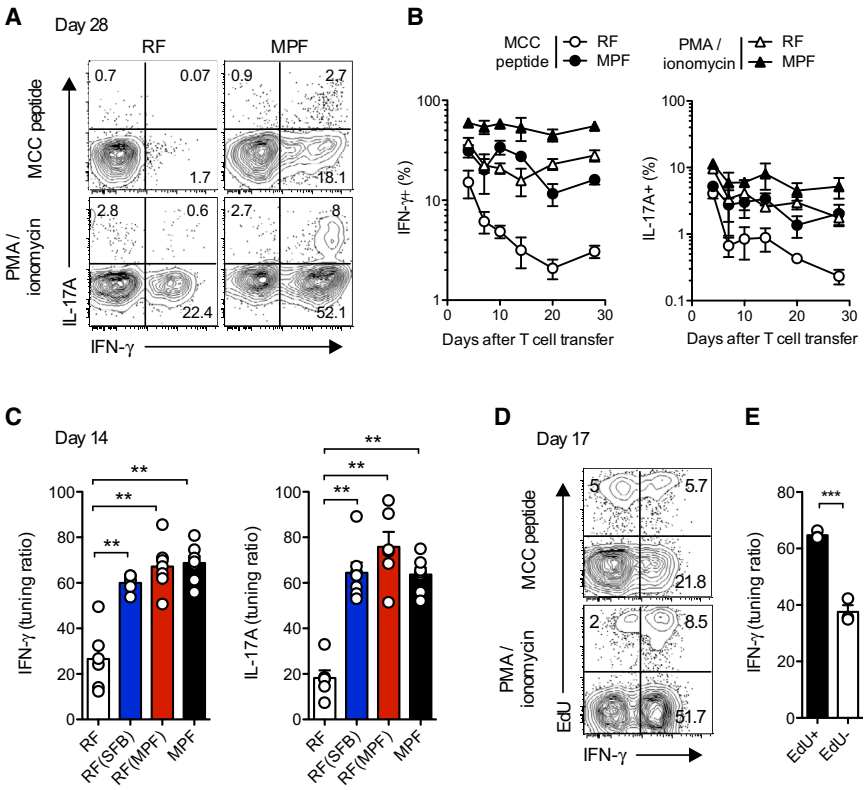


**Figure 5. Modulation of T Cell Tuning by Commensal Flora Broadly Affects Self-reactive CD4$^+$ Th1 and Th17 Subsets**

(A and B) Representative expression profiles at day 28 (A) and frequencies of IFN-γ$^+$ and IL17A$^+$ cells in live CD4$^+$Vβ3$^+$ T cells isolated from mLNs of mPCC,*Cd3e*$^{-/-}$ hosts at the indicated time points after naive 5C.C7 T cell transfer and restimulated for 3 hr with PMA and ionomycin or 3 μM MCC peptide (B). Data (mean ± SEM) were pooled from three independent experiments (n = 6–7 mice per group).

(C) Tuning ratios, as described in the Experimental Procedures, for IFN-γ and IL17A in live CD4$^+$Vβ3$^+$ T cells isolated from mLNs of indicated hosts 14 days after T cell transfer and restimulated for 3 hr with PMA and ionomycin or 3 μM MCC peptide. Data (mean ± SEM) were pooled from three independent experiments (n = 6–7 mice per group).

(D and E) Seventeen or 26 days after T cell transfer, MPF-housed mPCC,*Cd3e*$^{-/-}$ hosts were injected with EdU and sacrificed 1 hr later. Representative EdU and IFN-γ expression profiles in live CD4$^+$Vβ3$^+$ T cells isolated from mLNs at day 17 are shown in (D). The tuning ratio for IFN-γ in live EdU$^+$ versus EdU$^-$ CD4$^+$Vβ3$^+$ T cells is shown in (E). Data (mean ± SEM) were pooled from two independent experiments (n = 3 mice per group).

Furthermore, they demonstrate that the local microenvironment of an MPF-housed host's mLNs can actively induce the local reactivation of previously fully tuned self-reactive T cells and can lead to restored proliferative and effector functions.

## Inflammatory Gut-Microbiota-Derived Signals Impact Self-reactive T Cell Chronic Activation

Changes in the local microenvironment of MPF-housed gut-associated tissues could result from T-cell-dependent signals linked to enhanced IFN-γ production (Shalapour et al., 2010) and/or the appearance of autoimmune inflammatory symptoms. IFN-γ production by T cells, however, did not seem to play a critical role given that *Ifng*$^{-/-}$ 5C.C7 T cells responded identically to wild-type T cells in the MPF-housed host's mLN microenvironment (Figures S5A and S5B). Similarly, the impact of the gut microbiota on chronically activated 5C.C7 T cells appeared unaffected by the absence of B cells and overt autoimmune manifestations (Singh et al., 2006), as observed upon transfer into mPCC,*Rag2*$^{-/-}$ hosts (Figures S5C–S5G). Subsequent transfer of naive B cells further demonstrated the enhanced B cell helper and arthritis-inducing potential of chronically stimulated 5C.C7 T cells in MPF-housed T- and B-cell-deficient *Rag2*$^{-/-}$ hosts (Figure S5H). These results thus prompted us to look for an impact of the gut microbiota on the mLN microenvironment during the chronic phase of T cell activation.

Direct modulation of antigen presentation in mLNs did not seem to play a critical role in our model (Figure S4). Therefore, we investigated whether these effects could be the result of direct signaling of chronically activated T cells by one or more commensal-flora-induced molecules. Analysis of the expression pattern of over 30 cytokines in the serum of MPF- versus RF-housed hosts enabled us to identify four (G-CSF, IL-1Ra, IL-6, and IL-12p40) as significantly increased in host sera during the course of an MPF-induced chronic autoimmune T cell response (Figure 7A). Both IL-6- and IL-23-mediated signaling can affect T cell long-term proliferative responses, as evidenced in murine colitis models (Tajima et al., 2008; Ahern et al., 2010). To test their role in our model, we treated MPF-housed hosts with neutralizing mAb between days 7 and 14. In vivo neutralization of IL-12p40, but not IL-6, in an MPF-housed host was sufficient to reduce ongoing proliferation of and prevent the secondary CD25 expression normally observed in MPF-mLN T cells (Figures 7B and 7C and S6A and S6B). Similarly, analysis of the IL-2, IL-17A, and IFN-γ expression profiles and related tuning ratios confirmed a nonredundant role for IL-12p40, but not IL-6, in maintaining TCR responsiveness of chronically stimulated T cells, in addition to their Th1 and Th17 differentiation programs (Figures 7D and 7E and data not shown).

IL-12p40 is an integral part of IL-12p70 and IL-23 heterodimers. Interestingly, IL-12p70 directly induces CD25 expression in a STAT4-dependent manner and acts as a secondary signal for proliferation of CD4$^+$ Th1 cell clones (Yanagida et al., 1994; Nishikomori et al., 2002; O'Sullivan et al., 2004). Consistent with this, both RF and MPF mLN T cells showed responsiveness to IL-12p70 stimulation in vitro, as measured by CD25 upregulation (Figure S6C). The frequency of responding cells, however, appeared greatly increased in MPF-mLN T cells, in line with the increased Th1-driven response in MPF-housed hosts. As expected, IL-12p70 had no direct impact on naive T cells, and IL-23 did not induce any CD25 expression above

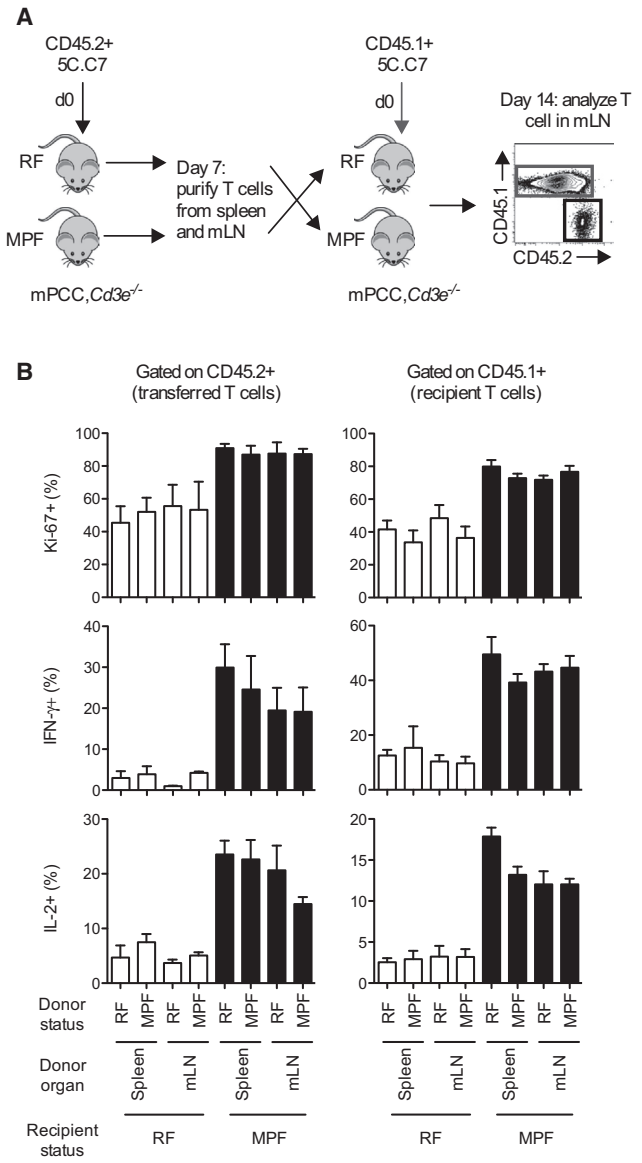


**Figure 6. Host Microbiota Dynamically Regulates Self-reactive T Cell Tuning**

(A) Two cohorts of mPCC,*Cd3e*$^{-/-}$ mice were injected simultaneously with either naive CD45.2$^+$ 5C.C7 T cells or CD45.1$^+$ 5C.C7 T cells. Seven days later, spleens and mLNs were harvested from hosts injected with CD45.2$^+$ T cells. Purified T cells ($10^6$) were further reinjected into the hosts previously transferred with CD45.1$^+$ T cells. mLN cells were stained at day 14 for Ki-67 expression or for intracellular IL-2 and IFN-γ after 3 hr stimulation with 3 μM MCC peptide (MCC).

(B) Frequency of Ki-67$^+$ (top), IFN-γ$^+$ (middle), or IL-2$^+$ (bottom) in live CD4$^+$Vβ3$^+$ T cells in the CD45.2$^+$ donor (left) or the CD45.1$^+$ recipient (right) fractions. Data (mean ± SEM) were pooled from three independent experiments.

See also Figure S5.

that of the medium control in any of the T cell populations tested (Figure S6C). More importantly, IL-12p70, but not IL-23, was able to enhance pS6 activation in MPF mLN T cells in response to low levels of MCC peptide in vitro (Figure S6D). As observed for CD25 upregulation, IL-12p70 also impacted pS6 activation in RF mLN T cells, albeit at much lower levels (data not shown). These results thus suggest IL-12p70 signaling as a potential key promoter of chronic self-reactive T cell responses in this murine model of autoimmune arthritis.

## DISCUSSION

Taking advantage of the commensal-flora-driven nature of a T-cell-transfer model of autoimmune arthritis, we revisited in this study the impact of commensal-flora-derived signals on the responses of self-reactive CD4$^+$ T cells to chronic self-presentation in vivo. As expected from recent studies in other autoimmune models (Wu et al., 2010; Lee et al., 2011), effects on the maintenance of, but not the early induction of, Th17 cells were observed. In vivo neutralization experiments with an anti-IL-17A monoclonal Ab, however, did not reveal any role for such cells in our model (unpublished data). Instead, transfer of *Ifng*$^{-/-}$ T cells clearly demonstrated the key pathogenic role played by IFN-γ-producing Th cells in this arthritis model. This observation further extends the scope of autoimmune pathologies that can be influenced by colonization of host gut-associated bacterial niches with specific member(s) of the gut flora, such as segmented filamentous bacteria, and all subsequent changes that it might induce for other commensal bacteria or viruses (Kane et al., 2011; Kuss et al., 2011).

How do SFB regulate IFN-γ production by chronically stimulated 5C.C7 T cells in mPCC,*Cd3e*$^{-/-}$ hosts? SFB reproducibly enhanced Th1 differentiation of autoreactive T cells, reminiscent of observations made in other models of autoimmunity (Wu et al., 2010; Lee et al., 2011). Observed as early as day 4 after T cell transfer and quite stably up to day 57 in both the spleen and mLNs, this effect of the gut flora, however, only accounted for a 1.6-fold (day 4) to 2-fold (day 14 and up) increase in the frequency of IFN-γ-producing T cells in MPF-housed hosts. Indeed, the major impact of the gut flora in this model system was observed during the chronic phase of the autoimmune T cell response, a phase during which the SFB-induced cytokine milieu locally favored the maintenance of both the sensitivity and the proliferative response of autoimmune CD4$^+$ T cells; this process is normally controlled by the T-cell-intrinsic down-regulation of TCR activation thresholds naturally occurring under chronic TCR activation, which we refer to as T cell tuning (Tanchot et al., 2001; Singh and Schwartz, 2003). Taken all together, these effects led to close to a 50-fold increase in the absolute number of recovered 5C.C7 T cells demonstrating IFN-γ-producing capacity after TCR restimulation on day 14 in the mLNs of SFB-harboring hosts.

It had been previously suggested that gut-draining lymphoid organs serve as the starting place of systemic T cell responses (Wu et al., 2010; Lee et al., 2011). In mPCC,*Cd3e*$^{-/-}$ hosts, however, effects on T cell activation appeared mostly limited to the mLNs, although small differences in T cell proliferation and sensitivity were detected as far away as the spleen later in the response (unpublished data). These effects, however, preceded a similarly localized enhanced B cell response and led a 10-fold increase in the absolute number of germinal center B cells by day 14 and plasma cells by day 21 in the mLNs (unpublished data). Although an input from the later splenic responses

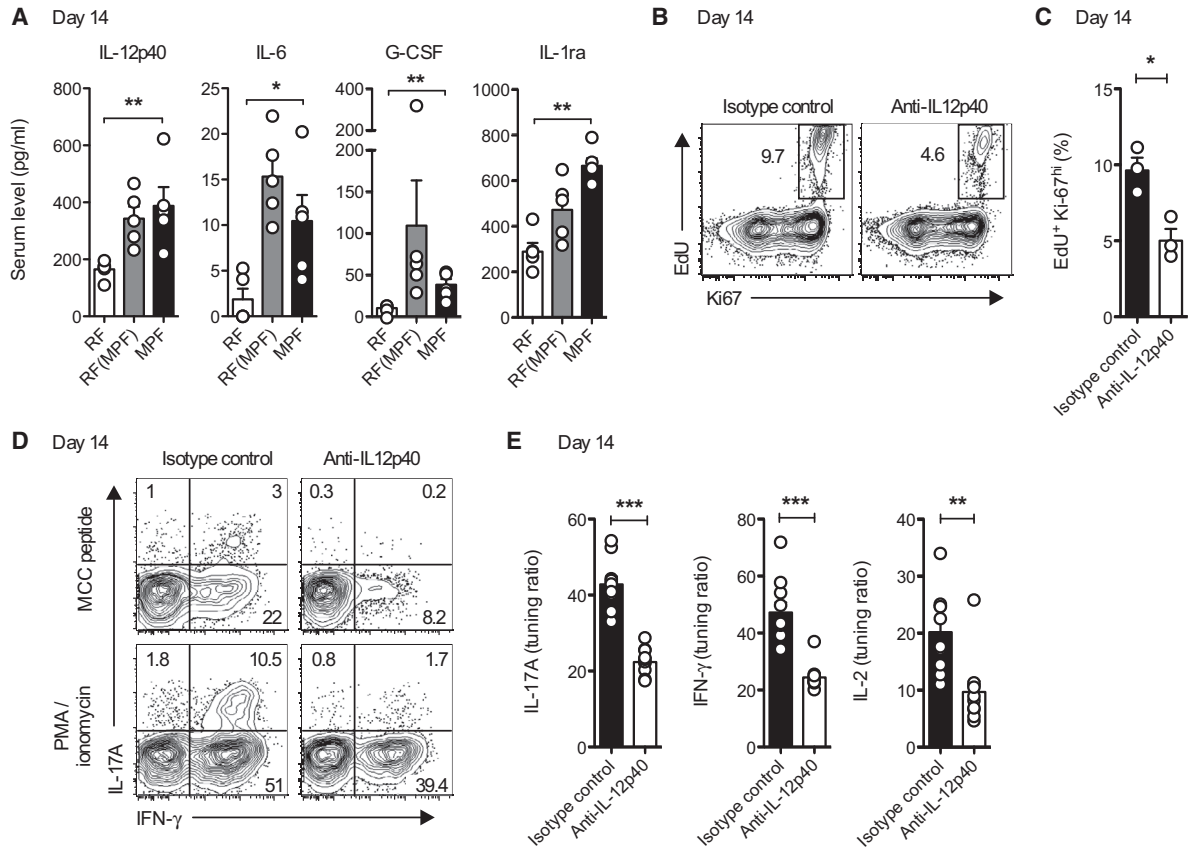


**Figure 7. In Vivo IL-12p40 Blockade Restores Optimal T Cell Tuning in MPF-Housed Hosts**

(A) IL-12p40, IL-6, G-CSF, and IL-1Ra levels in sera of mPCC,*Cd3e*$^{-/-}$ hosts 14 days after naive 5C.C7 T cell transfer. Data (mean ± SEM) were pooled from two independent experiments (n = 5 mice per group).

(B–E) Starting at day 7, MPF-housed mPCC,*Cd3e*$^{-/-}$ mice were further injected intraperitoneally twice daily with anti-IL12p40 or a corresponding isotype control mAb. At day 14, mice were injected with EdU and sacrificed 1 hr later. Representative EdU and Ki67 expression profiles are shown in (B), and the frequency of EdU$^{+}$Ki-67$^{hi}$ cells in live CD4$^{+}$Vβ3$^{+}$ T cells isolated from mLNs (n = 3 mice per group; mean ± SEM; *$p < 0.05$, Student t test) is shown in (C). Representative IL-17A and IFN-γ expression profiles are shown in (D), and the tuning ratios for IL-17A (left), IFN-γ (middle), and IL-2 (right) production in live CD4$^{+}$Vβ3$^{+}$ T cells isolated from mLNs at day 14 and restimulated for 3 hr with PMA and ionomycin or 3 μM MCC peptide are shown in (E). Data (mean ± SEM) were pooled from four independent experiments (n = 9–10 mice per group).

See also Figure S6.

cannot be excluded (Maccioni et al., 2002), it is highly probable that these locally enhanced T and B cell responses drive the detectably higher levels of high-affinity autoantibodies in the serum of MPF-housed hosts as early as day 21; this is a prerequisite for the induction of the arthritis pathologies (Singh et al., 2006).

The most interesting finding from this study is the demonstration that SFB colonization of host microbiota can locally enhance T cell responsiveness to endogenous antigens; this was recently suggested for macrophage responsiveness to IFN (Abt et al., 2012). T cell sensitivity has been previously described to be dynamically tunable to the level of antigen in the host (Tanchot et al., 2001; Singh and Schwartz, 2003; Han et al., 2010) or the T cell density (Tanchot et al., 2001). Detailed ex vivo analysis, secondary T-cell-transfer experiments, and in vivo IL-12p40-blockade experiments clearly demonstrated here that T cell sensitivity can be further modulated by flora-derived inflammatory signals. The ability of the inflammatory milieu, particularly IL-12p70, to imprint the sensitivity of individual T cell clones at the level of proximal TCR signaling pathways has recently been shown for CD8 T cells (Raué et al., 2013; Richer et al., 2013). Our data further extend this role to autoreactive CD4$^{+}$ T cells in the context of gut-associated lymphoid organs. Although we cannot fully exclude other independent regulatory mechanisms, it is tempting to speculate that chronic proliferation is an expected consequence of the maintenance of 5C.C7 T cell responsiveness to the endogenous level of PCC by the flora-specific inflammatory milieu. This would provide a conceptual framework to explain how chronic infections can sustain an ongoing immune response while autoantigens normally cannot.

In CD8 T cells, IL-12p70 can sustain mTOR activation (Rao et al., 2010), a key step in preventing the induction of clonal anergy in vitro (Allen et al., 2004; Zheng et al., 2007). We report here that IL-12p70, but not IL-23, also actively signals in tuned 5C.C7 CD4$^{+}$ T cells and leads to a similar enhancement of mTOR activation in response to low levels of MCC peptide stimulation. The enhanced in vitro responsiveness of MPF mLN

T cells to anti-CD3 stimulation suggests, however, a process that goes beyond simply providing a third signal during T cell activation. The T cell phenotype observed in this study could thus reflect a direct targeting by IL-12p70, through mTOR, of the expression and/or maintenance of the yet uncharacterized "anergic factors" associated with tuning. Alternatively, it could be an indirect consequence of an IL-12p70-induced, enhanced proliferation of the chronically stimulated T cells; this proliferation could itself be responsible for diluting out these "anergic factors," as once proposed for clonal anergy (Powell et al., 2001). A more detailed analysis of the molecular profile of tuned T cells in response to IL-12p70, or other cytokines, in combination with in vivo genetic experiments, is needed for fully deciphering the molecular mechanisms in play here.

One interesting question that remains is whether similar mechanisms are at play in endogenous T cells residing in gut-associated lymphoid organs and could explain the broad impact that SFB have on the maturation of the murine gut-adaptive immune system (Talham et al., 1999; Gaboriau-Routhiau et al., 2009; Chung et al., 2012). Numerous tissue- and APC-derived signals, such as pathogen-derived IL-12p70, IL-18, and IFN-I (Wang et al., 2012; Raué et al., 2013; Richer et al., 2013), gut-derived retinoic acid (Hall et al., 2011) and IL-23 (Ahern et al., 2010), or skin-derived IL-1 (Naik et al., 2012), can locally impact various aspects of $CD8^+$ or $CD4^+$ T cell fitness, although T cell sensitivity has never been carefully analyzed so far. Experiments done here in T-cell-replete mPCC,$Cd3e^{+/+}$ hosts revealed a striking similarity between the effects of the MPF gut flora on the proliferative response of chronically stimulated 5C.C7 and endogenous $CD4^+$ T cells. T-cell-extrinsic mechanisms did, however, limit the strength and location of this proliferative response—differences could only be detected in PPs—and severely blunt autoreactive T cell differentiation toward the Th1 lineage. Understanding the functional redundancy between the extended family of inflammatory signals and whether such signals can locally impact the sensitivity of various self- and non-self-specific T cells will clearly require further studies but could be key in understanding the general impact of the gut flora on the adaptive immune system.

In the case of autoimmune disease, dynamic regulation of T cell sensitivity represents a built-in negative-feedback mechanism designed to prevent overexuberant autoreactive T cell responses, a key tenant of the tunable-activation-threshold model (Grossman and Paul, 1992, 2001). Such negative regulation of T cell responsiveness over time has also been described in various chronic pathogen or tumor models (Carmichael et al., 1993; Rehermann et al., 1996; Oxenius et al., 1998; Staveley-O'Carroll et al., 1998; Zajac et al., 1998), two disease states in which it prevents full eradication of an unwanted target by the immune system. Unraveling the molecular mechanisms at play in bypassing this mechanism will be of key interest for helping design clinical approaches aimed at locally manipulating chronic T cell responses.

## EXPERIMENTAL PROCEDURES

### Mice

Mice used in this study have all been described previously (Tanchot et al., 2001; Singh et al., 2006). All colonies used in this study were bred under the National Institute of Allergy and Infectious Diseases (NIAID, National Institutes of Health [NIH]) contract at Taconic Farms, an AAALAC-accredited facility, in a RF-run barrier unit (IBU37), except for one colony of B10.A mPCC,$Cd3e^{-/-}$ mice bred in a MPF-run barrier unit (IBU40). Both Taconic RF and MPF barrier units are regularly monitored for known murine pathogens. In addition, the Taconic RF health standard excludes specified unacceptable bacteria, of which only *Klebsiella oxytoca* could be tested for in our MPF colonies (www.taconic.com/Health_Reporting). Mice were then housed in microisolator cages for the length of our experiments in a NIAID-run AAALAC-accredited facility. All experiments in this study were performed in accordance with the guidelines of the NIH Animal Care and Use Committee.

### Arthritis Experiments, Cohousing, and Antibiotic Treatments

The arthritic score used in this study has been described previously (Singh et al., 2006). Detailed experimental procedures for autoantibody titration are given in the Supplemental Information.

For cohousing experiments, mice were mixed upon arrival at our NIAID facility. For associating RF-housed mice with SFB, the mice were cohoused with SFB-monoassociated germ-free C57BL/6NTac mice for 3 weeks. At that time point, SFB reconstitution was confirmed by quantitative PCR of fecal 16S rDNA, and mice showing SFB titers similar to those of MPF-housed mice were injected with T cells and then further cohoused with mice displaying lower SFB titers. Detailed experimental procedures for SFB quantification and monoassociation of germ-free mice are given in the Supplemental Information.

For antibiotic treatments, freshly diluted combinations of 1 mg/ml each of ampicillin (sodium salt), neomycin sulfate, and metronidazole and 0.5 mg/l vancomycin hydrochloride (all from Sigma) were added to the drinking water and changed weekly (Ivanov et al., 2008). IL-12p40 was neutralized in vivo by the injection of 0.5 mg of mAb C17.8 at day 7 and 0.25 mg every other day until day 14 and was compared to control rat IgG2a (2A3) (all BioXCell). Cytokines in serum were analyzed with the Ray Biotech Quantibody mouse array (Ray Biotech).

### Cell Isolation, Adoptive Transfer, and Ex Vivo Analysis of T Cells

Naive 5C.C7 T cells were isolated from B10.A 5C.C7 TCR-transgenic $Rag2^{-/-}$ mice, and $0.5 \times 10^6$ to $1 \times 10^6$ cells were injected intravenously into the indicated hosts. Tuned 5C.C7 T cells were isolated from B10.A mPCC, $Cd3e^{-/-}$ and B10.A mPCC,$Cd3e^{+/+}$ hosts, restimulated in vitro, and stained as previously described (Singh and Schwartz, 2003; Singh et al., 2006). We calculated the tuning ratio for individual cytokines by dividing the frequency of individual cytokine producers obtained under MCC-peptide-mediated stimulation by the frequency of cytokine producers obtained under PMA- and ionomycin-mediated stimulation and presented it as a percent. Detailed experimental procedures are in the Supplemental Information.

### Statistics

All statistical analyses were performed with GraphPad Prism Software version 5 (GraphPad). The minimal level of confidence at which the result was considered significant was $p < 0.05$. For all kinetic experiments, statistical significance between each group over time was determined with a two-way ANOVA test. For all other statistical analysis, Mann Whitney nonparametric tests were used unless otherwise noted. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2013.06.005.

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